

**REMARKS**

In reviewing the previously filed Amendment, it was noted that clerical errors occurred in copying the claims. Therefore, to address some of those issues, claims were rewritten to avoid confusion. In other of the claims, the errors occurred in portions of the claims that were deleted or withdrawn from consideration.

The undersigned is sorry for the confusion, and hopes that the above amendments bring the claims in line with the form in which they were originally presented and subsequently amended.

No new matter was introduced by the above amendments to the specification and claims and thus, entry thereof is requested respectfully.

I. In item 6, the specification was objected to because of the hyperlinks at page 40, lines 2 and 30.

The specification was amended to remove the hyperlinks from page 40.

Also, the sequence identifiers from the listing were imported into the specification at page 46.

Hence, the objection can be withdrawn.

II. As to the drawing issue raised in item 7, complying formal drawings addressing the issues raised in the Form PTO 948 dated 3 March 2003 were filed 17 February 2004.

III. Regarding item 8, the claims now are in a format acceptable under U.S. practice and thus, the objection can be removed.

IV. As to item 9, while "s" is considered phonetically to have a vowel sound, claims 27 and 63, as well as claims 96 and 97, were amended as requested by the Examiner. Thus, the objection can be removed.

V. As to item 10, claim 18 has been re-presented as an independent claim. Thus, the objection can be withdrawn.

VI. Regarding item 11, while viewed unnecessary, because equivalent, claim 19 was amended as requested by the Examiner. Accordingly, the objection can be removed.

VII. In reference to item 12, the claims were amended to address the formality raised by the Examiner. Hence, withdrawal of the objection is in order.

VIII. In item 14, claims 18-35, 37, 39-52, 56-66 and 70-97 were rejected under 35 U.S.C. §112, first paragraph as allegedly being non-enabled.

The basic reasoning offered by the Examiner focuses on scope, as suggested by the use of the term "any" to characterize the various elements recited in the claims.

The rejection is traversed for the following reasons.

The heart of the instant invention relates to antibodies that specifically bind to the extracellular domain of the human zeta chain. The instant specification teaches a reproducible method that enabled for the first time, antibodies that specifically bind to the 11 amino acid extracellular domain of zeta on human cells. The instant specification teaches various known uses of antibodies to exploit the unique and inventive antibodies.

Each antibody, fragment or derivative thereof contains a variable region wherein at least one CDR is defined by sequence and/or function (i.e., the scope is not any and all antibodies but only those that bind to the extracellular domain of human zeta). That functional molecules selective for antigen can comprise isolated variable regions of immunoglobulins (i.e., may possess only single CDRs) may be seen, for example, in Laune et al., J Biol Chem (1997) 272(49):30937-30944, of record, and Feng et al., JBC (1998) 273(10):5625-5630, copies attached hereto for the convenience of the Examiner.

The specification teaches a reproducible method for making the antibodies of interest and contains examples illustrating various antibody constructs, including cloning of variable regions, identification of CDRs and expression of fragments (e.g., expression of Fab fragments, see Example 6) in a variety of known contexts, such as bispecific antibodies.

The antibody art is well known, the ways to manipulate and to use antibodies are known, and the portions of the claimed invention, outside of the antibodies of interest, are known. Thus, many of the points raised by the Examiner are non-issues. The instant application clearly teaches how to make the particular antibodies of interest.

The amount of experimentation required in view of the disclosure is routine (i.e., all of the methods needed to practice the invention are well known and the level of skill in the art at the time the application was filed is one which is ordinary).

Regarding "different molecules", "different cells", "second specificities", "intact cell", "molecules on cells" and so on, for example, for bi-specific antibodies, the identification of secondary cells, secondary molecules or secondary targets (primary target being the human zeta-chain) are elements within the skill of the artisan. Thus, an artisan would opt for using known targeting molecules for the second specificity of a bispecific molecule of interest. These issues relate but to a design choice.

Regarding claims 39, 45 and 46 that the Examiner believed are broader than the disclosure, the 11 amino acid extracellular domain of human zeta chain is the particular determinant of interest, for that is what is bound by the antibodies of interest. The instant specification teaches reproducible, enabled methods for making such antibodies, heretofore an unachievable goal. The antibodies of interest bind to the determinant on intact cells, rather than on isolated zeta chain, as had been achieved in the art.

A derivative of interest can be one that has at least one CDR of interest that confers specificity of the polypeptide containing same for the zeta extracellular domain. As known in the art, manipulation of the framework or sections flanking the CDR can be substantial so long as the at least one CDR is enabled to bind to the extracellular domain of the human zeta chain.

Regarding the "open" language and the 11 amino acid peptide, It is known that undecapeptides per se are not immunogenic. The art recognizes haptens can be conjugated to carrier molecules and can be immunogenic. Thus, it is of no moment as to what may be added to the 11-residue molecule, the goal is to obtain antibodies that specifically bind to the 11-residue molecule on intact cells, which has

been achieved in the instant invention.

The instant specification provides a thorough teaching of how to test antibodies to determine if they are specific for the extracellular domain of the human zeta chain as recited in the claims. Moreover, there are a number of known assays that can be used to determine the requisite specificity of an antibody of interest.

Within the ambit of the invention of interest are the various uses of the antibodies of interest. Such uses and the materials to enable those uses are known in the art or are design choices of the artisan practicing the instant invention.

For example, one of the uses of an antibody of interest is prophylactic administration. As with any other drug, an antibody can be given to prevent pathological states expected to occur. For example, when contemplating a bispecific receptor, the artisan will select a molecule known to bind to the second specificity selected for the pathological state of interest. Thus, the issue is not one of enablement because the artisan will choose a molecule of known function.

Thus, the proper inquiry of enablement is directed to antibodies to the extracellular domain on human zeta, and that inquiry will reveal that the instant specification and claims are enabled fully. Many other aspects of the claimed invention relate to uses of the antibodies of interest, and many of those are known in the art. Thus, a prima facie case of non-enablement has not been made and the rejection should be removed.

IX. Claims 18-35, 37, 39-52, 56-66 and 70-97 also were rejected under 35 U.S.C. §112, first paragraph as allegedly lacking written description.

The rejection is traversed for the following reasons.

Again, the basic reasoning offered by the Examiner focuses on scope, as suggested by the use of the term “any” to characterize the claim elements in the “alleged” showing to support the position advanced by the Examiner. .

The instant specification explicitly describes making the particular antibodies of interest, and the uses thereof, many of which are known in the art, relying on materials known in the art. An artisan will recognize that for the first time, an antibody to the extracellular domain of human zeta on cells was made. The specification provides details of methods for making an antibody of interest, such as that set forth in claim 39. An exemplified antibody was tested, cloned and sequenced, as well as manipulated to obtain fragments and derivatives thereof that bind to the extracellular domain of the human zeta chain.

An artisan would well recognize that applicant was in full possession of the claimed invention at the time the application was filed. That inescapable conclusion derives from the instant specification and the state of the art. The instant specification provides an enabled and reproducible teaching of how to make and how to use an antibody of interest. That one antibody is exemplified is no indication that the making of an antibody of interest is not described adequately.

The standard for the written description requirement is whether an artisan would recognize possession of the claimed invention by the inventor when the application describing same was filed. Applicant will be providing very soon a Declaration of one or ordinary skill in the art that will speak to that standard.

Moreover, there is no evidence presented that an artisan would not recognize that applicant has developed a way to make antibody to the extracellular domain of human zeta, and how to use same.

The claims would put in possession of the public what the Applicant claims as

the invention, thereby satisfying the statutory requirements of patentability.

For these reasons, it is believed a prima facie case of inadequate written description has not been made and the rejection should be removed.

X. Claims 23, 26, 29, 31, 34, 35, 40-44 and 48-51 were rejected under 35 U.S.C. §112, second paragraph for allegedly being indefinite.

The rejection is traversed for the following reasons.

To advance prosecution, claim 23 was amended to recite cell surface molecules other than zeta on a second intact cell.

As to claim 29, the second specificity is one selected by the artisan using any selected binding partner in the context of a bispecific molecule, wherein one specificity is to the human zeta extracellular domain. The second specificity is within the skill of the artisan using known materials and methods, as a design choice.

The skilled artisan would know the metes and bounds of a claim term and the claims per se. Thus, the rejection can be removed.

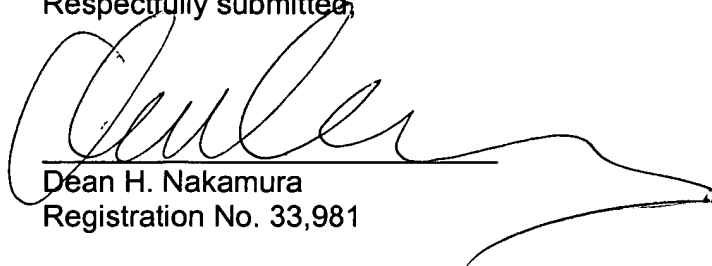
### **CONCLUSION**

Applicant submits that the pending claims are in condition for allowance. Reexamination, reconsideration, withdrawal of the objections and rejections, and early indication of allowance are requested respectfully. If any questions remain, the Examiner is urged to contact the undersigned at the local exchange noted below.

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Application No. 09/743,482  
Attorney Docket No. 105032.991240

If any fees are found to be applicable, please charge any additional fees or  
make any credits to Deposit Account No. 07-1896.

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**REPLACEMENT PAGE**

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nucleotide information was received from the Genebank database (<http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/>) for the kappa chain: Shepard and Gutman, Accession No. J02574 and for the mu chain: Parker, K.E., Accession No. X68312. The first strand of cDNA was then poly-G tailed using terminal transferase (Pharmacia, Freiburg) according to standard protocol. The tailed cDNA was PCR-amplified using a sense primer containing a poly-C stretch, based on the anchor primer sequence published by Gilliland, L. K. et al., (Tissue Antigens 47, 1-20, 1996) and designated 5'-AncTail (CGTCGATGAGCTCTAGAATTCCCCCCCCCCCCCD). This anchor primer was combined with an antisense primer, specific for the nucleotide sequence encoding the C-terminus of the kappa light chain constant region or that of the IgM-CH1 heavy chain domain, respectively. The primers were designated 3'ratck (GCGCCGTCTAGAATTAACACTCATTCTGTGAA) and 3'ratcmu (ATTGGGACTAGTCTCAACGACAGCTGGAAT). The PCR was carried out as follows: Primary denaturation: 94°C for 4 min.; 30 cycles of amplification: 93°C for 30 sec.; 55°C for 30 sec.; 72°C for 30 sec.; terminal elongation: 72°C for 3 min. Each of these primers contains a restriction enzyme cleavage site (5'-AncTail: *EcoRI*; 3'ratck: *XbaI*; 3'ratcmu: *SpeI*) which allows cloning of the corresponding PCR-fragments into a plasmid vector digested with *EcoRI/XbaI* or *EcoRI/SpeI*, respectively; for this purpose the bluescript KS+ plasmid vector (Genebank Accession No X52327) was used, since it also allows easy sequence analysis of the resulting inserts by using common sequencing primers. Due to an internal *SpeI* cleavage site within the variable region of the heavy chain (VH) partial digestion followed by cloning of the full length fragment was necessary to obtain the complete sequence information of the VH-domain. Partial digestion was carried out according to standard protocols (Sambrook, Cold Spring Harbour Laboratory Press 1989, second edition). Several clones of heavy and light chain fragments proved to have identical sequences, respectively, and could be identified to encode either functional VL- or VH-regions (see Figure 6 and 7).

The mature N-terminus of both variable chains was identified by comparing their sequences with those found in Genebank database (<http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/>) and subsequently a second set of PCR-primers was designed to introduce appropriate restriction enzyme cleavage sites in frame with the coding sequences of the 2-B-5 Fab-antibody fragment and with regard to the requirements for subcloning into a bacterial expression vector. The two

## Peptides Derived from the Complementarity-determining Regions of Anti-Mac-1 Antibodies Block Intercellular Adhesion Molecule-1 Interaction with Mac-1\*

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Peptides or small molecules that can block the interaction of the integrin Mac-1 with its receptor, intercellular adhesion molecule-1 (ICAM-1), have not previously been developed. We studied this interaction by measuring the adherence of ICAM-1-expressing Chinese hamster ovary (CHO) cells to immobilized, purified Mac-1. Nucleotide sequence information was obtained for the complementarity determining regions (CDRs) of three antibodies (44aach, MY904, and 118.1) shown to block Mac-1-mediated cell adherence. Peptides were synthesized based on the predicted amino acid sequences of the CDRs and tested for the ability to block cell adhesion to Mac-1. Peptides derived from CDR1 of 44aach, CDR2 of 118.1, and CDRs 1 and 3 of MY904 heavy chains were found to possess blocking activity at 10–100  $\mu$ M. This may indicate that one or two CDRs contribute disproportionately to the antibody binding affinity. The binding of ligands to Mac-1 has been shown to require a region of the  $\alpha$ -chain known as the I- or A-domain. We have recombinantly produced Mac-1 I-domain, and show that it is also capable of supporting the adherence of ICAM-1-expressing CHO cells. The adherence of ICAM-1-CHO cells to the I-domain is inhibited by 44aach and 118.1 and by the CDR peptides from 44aach and 118.1. By using phage display of peptide libraries based on the 118.1 CDR peptide with five residues randomized, we were able to identify a novel peptide inhibitor of Mac-1 with substitutions at all five positions. These peptides provide lead structures for development of Mac-1 antagonists.

The extravasation of white blood cells to sites of inflammation and the phagocytosis of opsonized microorganisms by these cells is clearly crucial to host defense. However, the mounting of an inappropriately large mobilization of phagocytic cells is thought to contribute to organ damage in sepsis, in adult respiratory distress syndrome, and following reperfusion of ischemic tissue (1, 2). Activated neutrophils are recruited into tissues or are sequestered in the microcirculation of the lung and liver; in either case tissue is damaged upon their degranulation and release of enzymes and activated oxygen species. Interruption of neutrophil extravasation or oxidative burst may be an effective means of damage control in these situations.

Mac-1 is a cell surface glycoprotein contributing to several

myeloid cell functions including adherence to and transmigration across the endothelium, binding and phagocytosis of opsonized particles, and the oxidative burst (3–5). It is a heterodimer of two transmembrane proteins, CD11b ( $\alpha_M$ ) and CD18 ( $\beta_2$ ), the latter also being part of the related integrins LFA-1, p150/95, and  $\alpha_X\beta_2$ . The major adhesion partner for Mac-1 is ICAM-1<sup>1</sup> (6), which is a member of the Ig supergene family and contains five extracellular Ig domains of the C2 type, characteristic of Fc receptors and proteins involved in cell adhesion (7). However, the complexity of the functions involving Mac-1 results in part from the fact that an array of ligands besides ICAM-1 is also recognized by this molecule, including iC3b, fibrinogen, and factor X (3).

The precise residues of Mac-1 and ICAM-1 mediating their interaction are not known, although the domains responsible have been elucidated. Ig domain 3 of ICAM-1 has clearly been implicated in binding to Mac-1, whereas domain 1 mediates binding to the related adhesion molecule, LFA-1 (8). CD11b contains a 200-amino acid “inserted domain” or “I domain,” so called due to its presence only in the other  $\beta_2$  integrins and in the VLA  $\alpha 1$  and  $\alpha 2$  subunits and its absence in most other integrins. Antibodies to this domain can block ICAM-1 binding, as well as that of iC3b and fibrinogen (3). Mutations within the I-domain of Mac-1 have been shown to prevent binding of ICAM-1 and iC3b (9–11). The Mac-1 I-domain has been expressed recombinantly, and there are some data suggesting that it interacts with fibrinogen, iC3b, and soluble ICAM-1 (4, 12). Whether the I-domain can support the adherence of ICAM-1-expressing cells independent of other domains of Mac-1 has not been previously demonstrated.

The lack of information on the precise sequences within ICAM-1 and Mac-1 that interact has precluded modeling of small molecule inhibitors. One approach to overcoming this problem that has proven successful in other cases has been to use CDR sequences from antibodies directed at the active site as lead structures. Antibodies useful in this regard have been either developed as anti-idiotypic to the ligand (13) or simply chosen by virtue of their ligand blocking activity (14). As well as providing lead inhibitors, in some cases CDRs have shared sequence similarity with a portion of the known ligand, implicating that sequence in receptor binding (14–16).

We have produced the Mac-1 I-domain recombinantly and show that it supports the adherence of ICAM-1-expressing

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<sup>1</sup> The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; CDR, complementarity-determining region; HBSS, Hanks’ buffered salt solution; HBSS+, HBSS with calcium and magnesium; HC, heavy chain; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; HSA, human serum albumin.

cells. Several antibodies that block the adherence of ICAM-1 to both Mac-1 and to the I-domain have been sequenced to allow determination of CDR structures. Peptides based on these CDR structures are shown to block the adherence of ICAM-1 to both Mac-1 and the I-domain.

#### MATERIALS AND METHODS

**Purification of Mac-1**—Peripheral blood leukocytes were purified from "Buffy coats" (Stanford Medical School Blood Center, Stanford, CA). Buffy coats were diluted 1:1 with HBSS and layered on Histopaque (Sigma) gradients as described (17). Both mononuclear and polymorphonuclear cell layers were collected, and red blood cells were lysed one or two times as necessary. Mac-1 was purified from lysates of the remaining leukocytes by immunoaffinity chromatography essentially as described by Diamond *et al.* (6) and purity assessed using SDS-polyacrylamide gel electrophoresis (see Fig. 1A).

**Construction of Mac-1 I-domain**—The construction of the I-domain was based on a plasmid construct reported by Michishita *et al.* (11). The I-domain of human Mac-1 from the glycine residue at position 111 to the alanine at position 318 was generated using synthetic oligonucleotides and PCR. Eight overlapping oligonucleotides were synthesized and combined in a stepwise PCR procedure to generate the final 603-base pair fragment. The 5'-most oligo included a *Bam*HI site present naturally in the Mac-1 gene sequence, whereas the 3'-most oligo included an added *Eco*RI site. The internal *Eco*RI site in this I-domain region was eliminated via a single base change from A to T at the third position in the glutamate codon at position 179 (a silent mutation). Each pair of oligonucleotides sharing partial complementarity was annealed and subjected to PCR (3' at 94 °C, followed by 10 cycles of 1' at 94 °C, 2' at 55 °C, and 3' at 72 °C) using 250  $\mu$ M dNTPs and VENT polymerase (New England Biolabs, Beverly, MA). The PCR products were then mixed, melted for 3' at 94 °C, and subjected to PCR as described above. After assembly of all of the oligos, the resulting *Bam*HI-*Eco*RI fragment was cloned into the pGEX-2T (Pharmacia Biotech Inc.) vector at the *Bam*HI and *Eco*RI sites, resulting in an in-frame fusion with an N-terminal domain encoding glutathione S-transferase.

**Purification of Recombinant I-domain**—The glutathione S-transferase-I-domain fusion protein was expressed in *Escherichia coli* cells (strain JM101). Overnight cultures of *E. coli* JM101 were diluted 1:10 with L broth medium containing ampicillin and grown for 1 h at 37 °C. Isopropyl- $\beta$ -D-thiogalactoside (1 mM) was added to induce expression of the fusion protein, and after 3 h of growth, bacteria were pelleted and frozen at -80 °C. Pellets derived from a 1-liter culture were then thawed and resuspended in 18 ml of cold PBS, pH 7.4, phenylmethylsulfonyl fluoride was added at 1 mM, and the samples were disrupted on ice at 10,000 p.s.i. in a French Press (SLM Instruments, Inc., Rochester, NY). 20% Triton X-100 was then added to a final concentration of 1%, and the lysate was incubated on ice for 30 min with occasional rocking. After centrifugation at 12,000  $\times$  g for 10 min, the supernatant was incubated with glutathione-Sepharose 4B (1.5 ml, Pharmacia) for 1 h at room temperature. The beads were then washed once in PBS plus 0.35 M NaCl and washed four times with PBS and were resuspended in 5 ml of PBS. 200 units of human thrombin (Enzyme Research Laboratories, South Bend, IN) was added to cleave the I-domain from the fusion protein, and the mixture was incubated for 2 h at room temperature. NaCl and MgCl<sub>2</sub> were added to the cleaved soluble recombinant I-domain at final concentrations of 0.35 M and 1 mM, respectively, and the samples were then passed through a 700- $\mu$ l benzamide-Sepharose 6B (Pharmacia) column to remove the thrombin. The flow-through was assayed for thrombin activity using Chromozym TH (Boehringer Mannheim, Indianapolis, IN) as a substrate, and the clearance was greater than 150-fold. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad). Typical yields of the recombinant I-domain were 3–5 mg/liter bacterial culture.

**Mass Spectrometric Analysis of I-domain**—Electrospray ionization was performed on a Finnigan SSQ 7000 mass spectrometer (San Jose, CA) in the positive ion mode. Liquid chromatography/mass spectrometry was performed using a capillary reversed phase column with a flow rate into the mass spectrometer of 5  $\mu$ l/min. To map tryptic peptides, electrophoresis of I-domain protein was carried out in 12% polyacrylamide followed by transfer to Immobilon (70 V, 60 min; Ref. 18). *In situ* tryptic digestion was carried out according to the method of Wong *et al.* (19). Capillary HPLC was performed using a Valco tee to split the 200  $\mu$ l/min flow from an HP 1090 HPLC PV5, driving the capillary system (Vydac C18 0.32  $\times$  250-mm column, maintained at 40 °C, Microtech Inc., Sunnyvale, CA) at 5  $\mu$ l/min. The gradient was as follows: 0 min, 100% A (0.1% trifluoroacetic acid in H<sub>2</sub>O); 40 min, 30% B (0.09%

trifluoroacetic acid in acetonitrile); 50 min, 60% B; 51 min, 0% B; 72 min, 0% B.

**Adherence Assays**—The adherence of human neutrophils to wells coated with human serum was carried out exactly as described previously (17). CHO cells were stably transfected with an expression vector encoding human ICAM-1 (20), and their adherence was assessed as follows. ICAM-1-CHO cells were grown in RPMI 1640 with 10% fetal bovine serum and used at 80% confluence. Cells were loaded with calcein-acetoxymethyl ester (Molecular Probes, Eugene, OR) at 5  $\mu$ M in HBSS++ for 30 min at 37 °C. The cells were then detached from the flask with PBS containing 5 mM EDTA (15 min, 37 °C), washed in HBSS++ containing 0.1% HSA (Sigma), and resuspended in HBSS++ containing 0.5% HSA at  $2 \times 10^6$ /ml. 96-well plates were coated with purified Mac-1 or I-domain in HBSS++ (50  $\mu$ l/well) for 2 h at 37 °C. In the case of Mac-1,  $\beta$ -octylglucoside at a final concentration of 0.15% was present in the coating solution. For each lot of Mac-1, the optimal protein concentration for coating the wells (5–13  $\mu$ g/ml) was determined as that which best supported the adherence of ICAM-1-CHO cells but not of vector-CHO cells. Wells were washed twice with HBSS++ containing 0.1% HSA and blocked with HBSS++ supplemented with 0.5% HSA for 30 min at 37 °C. Test compounds were preincubated in blocked wells for 15 min at 37 °C in 50  $\mu$ l, and then 50  $\mu$ l of cells were added for an additional 60-min incubation. Nonadherent cells were removed by gently inverting plates and blotting on paper towels. Wells were washed twice with HBSS++ containing 0.1% HSA. Adherent cells were quantitated in a 96-well fluorescence plate reader (IDEXX Labs, Westbrook, ME). HSA lots were tested in this assay to find those that gave a minimal background adherence and a maximal adherence to Mac-1. All incubations in HBSS++ were carried out without CO<sub>2</sub>.

**Monoclonal Antibodies**—Hybridoma cells producing the anti-Mac-1 monoclonal antibodies LM2/1 (murine IgG1), 44aabc (murine IgG2a  $\kappa$ ) and MY904 (murine IgG1  $\kappa$ ) were from ATCC (21, 22). The hybridoma cell line secreting mAb 118.1 (murine IgG1 $\kappa$ ) was generated from a Balb/C mouse immunized according to Diamond *et al.* (3). Spleen cells were mixed in a 5:1 ratio with FOXP-NY murine myeloma cells (ATCC 1732 CRL) and fused by slow addition of polyethylene glycol 1500 (0.5 ml/10<sup>6</sup> cells, Boehringer Mannheim). After 1 min at 37 °C, the cell suspension was slowly diluted in RPMI, centrifuged at 200  $\times$  g for 7 min, and resuspended in selection medium (RPMI 1640, 20% fetal bovine serum, Pen/Strep, AAT media supplement (Sigma), and STM Mitogen (R&B ImmunoChem Research Inc.)). After 9 days of growth in 96-well culture plates, the hybridoma supernatants were screened for antibodies binding to purified Mac-1 in an enzyme-linked immunosorbent assay adapted from Diamond and Springer (23). Serum-free conditioned media from the hybridoma lines were harvested from confluent roller bottles, and antibodies were purified by protein A chromatography using PROSEP-A affinity resin (BioProcessing, Ltd.) following the manufacturer's suggested protocol.

**Cloning and Sequencing of the Variable Regions of 44aabc, MY904, and 118.1**—mRNA was isolated from 44aabc, MY904, and 118.1 hybridoma cells (10<sup>6</sup> cells each, grown in RPMI 1640, 10% fetal bovine serum, 10 mM HEPES) using the Mini RiboSep<sup>TM</sup> mRNA Isolation Kit (Becton Dickinson, Bedford, MA). Following first strand cDNA synthesis using a cDNA synthesis kit (Amersham Corp.), V<sub>H</sub> and V<sub>L</sub> regions were amplified (Vent DNA polymerase, New England Biolabs, Beverly, MA) using the following primers: V<sub>H</sub> regions, upstream primer 5'-GCA-GAATTCGAGTSCARTTRCARCA and downstream primer 5'-GCA-GAATTCGGGGCCAGTGGATAGAC; V<sub>L</sub> regions, downstream primer 5'-GCAGAATTCGGTGGGAAGATGGATACAGTT coupled to upstream primers 5'-GCAGAATTCACMCARTCHCCAGTNAT (44aabc), 5'-GCA-GAATTCGAYATYGTBCTGACNCA (MY904), or 5'-GCAGAATTCGAY-GTBGKTATGACMCA (118.1) (S = C or G; R = A or G; M = A or C; Y = C or T; K = G or T; H = A, C, or T; B = C, G, or T; and N = A, C, T, or G). The sequences of the downstream primers were based on the conserved nucleotide sequences of the 5'-ends of the constant regions of the heavy and light chains. The upstream primers were designed from either the experimentally determined N-terminal amino acid sequences of the three antibodies or deduced N-terminal "consensus" amino acid sequences of murine immunoglobulin variable regions from GenBank<sup>®</sup>. The mouse codon usage was also considered when degenerate primers were designed. An *Eco*RI site was introduced at the 5'-end of all the primers to facilitate cloning. PCR was performed for 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, and bands were resolved by electrophoresis in a 1% agarose gel.

The correctly sized PCR products were digested with *Eco*RI, gel-purified, and ligated into *Eco*RI-digested pUC9 vector DNA. The ligated DNA was then transformed into *E. coli* JM101 by electroporation, and clones were selected on LB plates containing ampicillin. For each V<sub>H</sub> or

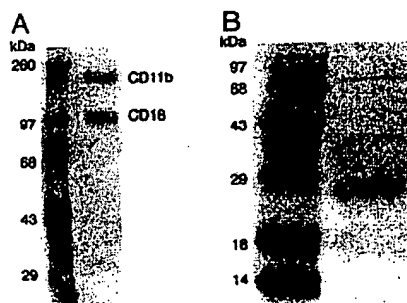


FIG. 1. Characterization of purified Mac-1 and I-domain. Purified Mac-1 (A) or Mac-1 I-domain (B) were subjected to SDS-polyacrylamide gel electrophoresis using 8 and 16% acrylamide, respectively. Prestained proteins used as standards were from Bethesda Research Technologies, Inc. (Gaithersburg, MD). Gels were stained with Commae Brilliant Blue.

V<sub>L</sub> region, three or four positive clones were picked, DNA was prepared using the Wizard miniprep system (Promega, Madison, WI), and the PCR product carried in the plasmid DNA was sequenced.

**Construction of Phage Libraries**—Two peptide libraries were constructed in which the library inserts were directly linked to the N terminus of the C-terminal domain of M13 gene III as described (24). The GYXDXYXGLXYN and GXLPXYXGXTYN libraries were prepared using synthetic oligonucleotides containing core sequences GGA TAT NNS GAT NNS TAC NNS GGT NNS ATT NNS TAC AAC and GGA NNS ATT NNS CCT NNS TAT NNS GGT NNS ACC TAC AAC, respectively. The nucleotides were made double-stranded by extension with Klenow polymerase (New England Biolabs) from a 5' primer. The resulting products were gel purified using MERmaid spin kit (Bio101 Inc., Vista, CA) and inserted into the *Bst*II and *Bam*HI sites of the phagemid vector pAL53.<sup>2</sup> The library-containing vectors were then transfected into *E. coli* Top10F' cells using 10–12 electroporations. The yields of the two primary libraries were  $0.5\text{--}1 \times 10^7$ .

**Panning of Phage Libraries**—Libraries were packaged into phage particles by infection of the transfected *E. coli* Top10F' library with M13K07 helper phage, resulting in expression of less than one copy of the peptide-gene III protein per phage (24). Phage expressing Mac-1 binding peptides were selected by panning on 96-well enzyme-linked immunosorbent assay plates (Corning) coated with Mac-1 (0.5  $\mu\text{g}/\text{well}$  in HBSS++ containing 0.15%  $\beta$ -octylglucoside for 2 h at 37 °C). After the wells were washed and blocked with HBSS++ supplemented with 0.5% HSA, 10  $\mu\text{l}$  of partially purified phage ( $\sim 5 \times 10^{11}$  cfu) was added to the wells in a total volume of 100  $\mu\text{l}$  of the same buffer and incubated at 37 °C for 2 h while shaking. The unbound phage particles were then removed by washing with 100  $\mu\text{l}$  of HBSS++/0.05% Tween six times quickly on ice. Bound phage were eluted with 200  $\mu\text{l}$  of 100 mM citrate buffer, pH 3.0/0.1% Tween for 30 min at room temperature with shaking and neutralized with 25  $\mu\text{l}$  of 1 M Tris base. The wells were then washed three times with 100  $\mu\text{l}$  of PBS, and eluates and washes were combined. The eluted phage were titrated and amplified and carried through three more rounds of enrichment by panning on Mac-1. The stringency of the PBS wash conditions was increased in successive rounds as follows: nine quick washes at room temperature in round 2, followed by two additional washes for 10 min each with shaking in round 3 and three washes for 10 min each with shaking in round 4. Phage were randomly selected for sequencing after round 4.

## RESULTS

**Production of Recombinant Mac-1 I-domain**—Purified Mac-1 has been shown to support the adherence of ICAM-1-expressing L-cells (6). To determine whether the I-domain of Mac-1 could similarly support the adherence of cells expressing ICAM-1, this domain was expressed recombinantly in *E. coli*. Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis showed a major band migrating as expected and in some cases a minor band with slightly slower migration (Fig. 1B). Analysis of the purified protein by mass spectrometry

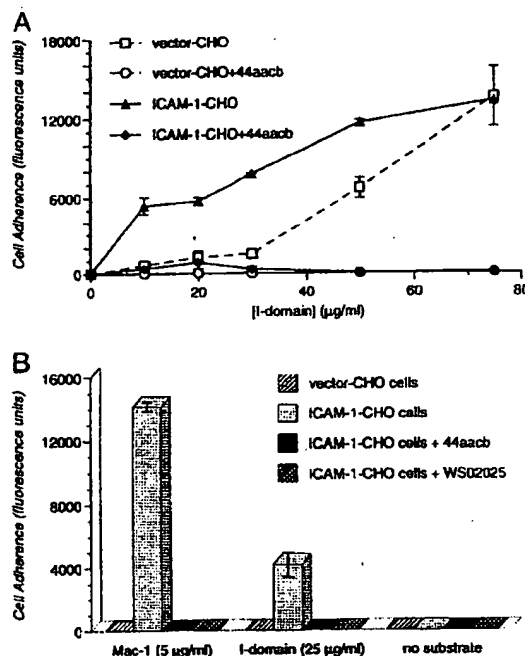


FIG. 2. Characterization of adherence assays based on purified Mac-1 or Mac-1 I-domain. The I-domain (A and B) or Mac-1 (B) was plated at the indicated concentrations, and the adherence of calcein-loaded ICAM-1-CHO cells or vector-CHO cells was quantitated as described under "Materials and Methods." Where indicated, plates were preincubated for 15 min with 10  $\mu\text{g}/\text{ml}$  of the blocking Mac-1 antibody, 44aabc. In some cases, cells were preincubated for 15 min with the anti-ICAM-1 antibody, WS02025. These data are the means of duplicate determinations ( $\pm$  individual values) and are representative of numerous experiments.

showed a major peak of 24,103 Da, in agreement with the molecular mass predicted for this construct. Also revealed was a minor peak of 25,102 Da, present at approximately 10–20% the level of the major peak in different preparations. To determine the source of the additional 1000 Da, the proteins in the I-domain preparation were separated by SDS-polyacrylamide gel electrophoresis and subjected to both N-terminal sequence analysis and tryptic peptide mapping. Both the 24- and 25-kDa proteins had the expected N-terminal sequence of GSNLRQQP. The 24- and 25-kDa bands showed a similar tryptic map pattern with the exception of the C-terminal peptide (IFANSS; mass, 638 Da), which disappeared from the digest of the 25-kDa band. A new peptide (mass, 1634 Da) was evident in the mass of the 25-kDa protein. The mass of the new peptide was 997 Da larger than the predicted C-terminal peptide, similar to the mass difference observed in the intact molecules, and thus we conclude that the added mass is contained at the C terminus. The 1634-Da peptide could be accounted for by translation of the usual stop codon in the pGEX-2T expression vector as a tryptophan residue, translation of 10 additional vector residues prior to the next stop codon, and proteolytic trimming of three C-terminal vector residues, resulting in the tryptic peptide IFANSSWLTDDLPR.

**Adherence Assays Based on Purified Mac-1 or the Mac-1 I-domain**—Purified recombinant I-domain was tested for the ability to support the adherence of ICAM-1-CHO cells. As shown in Fig. 2A, the I-domain did support the adherence of ICAM-1-CHO cells, in a concentration-dependent manner. For this particular lot of I-domain, 10–30  $\mu\text{g}/\text{ml}$  in the solution used to coat the wells resulted in good adherence of ICAM-1-CHO cells and low background adherence of vector-transfected

<sup>2</sup> D. L. Damm, B. L. Garrick, K. McFadden, D. D. Lesikar, A. B. Lucas, and R. T. White, manuscript in preparation.

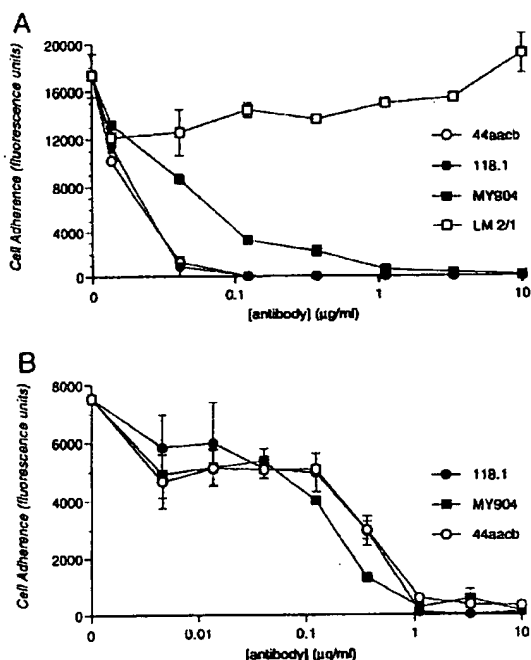


FIG. 3. Inhibition of ICAM-1-CHO cell adherence to Mac-1 or Mac-1 I-domain by anti-Mac-1 antibodies. Mac-1 (A) or the I-domain (B) was preincubated with the indicated antibodies for 15 min prior to addition of ICAM-1-CHO cells. These data are the means of duplicate determinations ( $\pm$  individual values) (A) or the means  $\pm$  S.E. of triplicate (B) determinations and are representative of over four experiments.

CHO cells. The adherence of ICAM-1-CHO cells was blocked by the anti-Mac-1 antibody 44aach. However, at high levels of I-domain, vector-transfected CHO cells were also adherent, and this adherence was blocked by 44aach. These results indicate that the I-domain of Mac-1 interacts not only with ICAM-1 but also with an unknown receptor on CHO cells. This interaction with a CHO cell receptor is not unique to the recombinant I-domain, because vector-CHO cells also adhered to high levels of purified Mac-1, and this adherence was similarly blocked by 44aach (data not shown).

To verify that under typical assay conditions with low substrate level, the observed adherence of ICAM-1-CHO cells was due to ICAM-1 rather than endogenous CHO receptors for Mac-1, a blocking ICAM-1 antibody was preincubated with cells prior to addition to the plate. This antibody completely blocked the adherence of ICAM-1-CHO cells to both the I-domain and to Mac-1 (Fig. 2B). The number of fluorescently labeled ICAM-1-CHO cells adhering to the I-domain under optimal conditions was typically 3–4-fold less than that adhering to Mac-1.

**Characterization of Anti-Mac-1 Antibodies**—The anti-Mac-1 antibodies 44aach and MY904 block the Mac-1-mediated adherence of neutrophils (17, 22). The antibody 118.1 was generated as described under "Materials and Methods" and also shown to block Mac-1-mediated neutrophil adherence (data not shown). 44aach, MY904, and 118.1 were found to bind to Mac-1 in an enzyme-linked immunosorbent assay format with  $EC_{50}$  values of 12.0, 9.3, and 10.7 nM, respectively. Preincubation of these antibodies with Mac-1 (Fig. 3A) resulted in inhibition of the adherence of ICAM-1-CHO cells with  $IC_{50}$  values of 50–500 pM in four assays. The three antibodies also blocked I-domain-mediated adherence (Fig. 3B) with  $IC_{50}$  values of 1–3 nM in four assays. LM 2/1, which does not block neutrophil adherence,

TABLE I  
CDR regions from anti-Mac-1 antibodies

	Amino acid sequences	$IC_{50}^a$ $\mu$ M
Heavy chain CDR1		
44aach	<u>YTFTNYWINVVKO</u>	33.2 $\pm$ 20 (5)
MY904	<u>YTFSNYWIETVVKO</u>	77.9 $\pm$ 19.6 (2)
118.1	<u>YSFTDYNMYVVKO</u>	>100 (2)
Heavy chain CDR2		
44aach	<u>ENIGNIYPSDTYINHNOKFKDKA</u>	>100 (1)
MY904	<u>ENIGEILPGSDSTNYNOKFKGKA</u>	>100 (1)
118.1	<u>ENIGYIDPYGGITYNOIFKGA</u>	30.4 $\pm$ 6.1 (4)
Heavy chain CDR3		
44aach	<u>AVVFCTRSAY...ANYFDYWGQG</u>	>100 (2)
MY904	<u>AVYFCARGGIIITAHYFDYWGQG</u>	106 $\pm$ 9.7 (4)
118.1	<u>AVYSCAIGGSYGWCS...YWGQG</u>	>200 (1)
Light chain CDR1		
44aach	<u>SFSCRASONIGTSL...HWYQQ</u>	>200 (1)
MY904	<u>TISCRASQSVSTSSY...SYMHWYQQ</u>	>200 (2)
118.1	<u>SISCRSSQSLVHNSNGNTYLHWYQQ</u>	NT <sup>b</sup>
Light chain CDR2		
44aach	<u>LLIKYASESISGIPS</u>	>200 (2)
MY904	<u>LLIKYASNLSEGVPA</u>	>100 (1)
118.1	<u>LLIKYVSNRFSGVDP</u>	NT
Light chain CDR3		
44aach	<u>DYYCOQSDSWPTLTFGAG</u>	>100 (2)
MY904	<u>TYVCOHSWEIP...LTFGAG</u>	>200 (1)
118.1	<u>VYFCSQSTHVP...FTFGGG</u>	NT

<sup>a</sup> Peptides (underlined portions) representing the CDR regions plus some flanking sequences were synthesized and tested for inhibition of ICAM-1-CHO adherence to Mac-1; data shown are mean  $\pm$  SDEV.

<sup>b</sup> NT, not tested.

does not block ICAM-1-CHO adherence to Mac-1 (Fig. 3A).

**Characterization of CDRs of Anti-Mac-1 Antibodies**—mRNA isolated from hybridoma cells producing 44aach, MY904, and 118.1 was used to determine the nucleotide sequence encoding the CDRs of each of the antibodies. The deduced amino acid sequences are shown in Table I. Peptides were synthesized corresponding to the underlined portions of the sequences in Table I. These peptides were tested for inhibition of ICAM-1-CHO cell adherence to Mac-1 (Table I). One or two HC CDRs from each antibody blocked Mac-1-mediated adherence with an  $IC_{50}$  at or below 106  $\mu$ M. Of the light chain CDR peptides made for antibodies 44aach and MY904, none possessed blocking activity. Two of the active HC CDR peptides were also tested for blocking activity in the adherence assay using the I-domain as a substrate. HC CDR1 from 44aach and HC CDR2 from 118.1 were found to possess comparable blocking activity in this assay (data not shown).

**Identification of Additional Blocking Peptides through Phage Display**—Variants of the 118.1 HC CDR2 blocking peptide were identified by displaying libraries based on this peptide on phage and selecting for those phage that bound to Mac-1. To select residues for randomization, the structure of 118.1 was modeled on the three-dimensional structure of the antibody D11.15, an anti-lysozyme antibody (Brookhaven Protein Data Bank 1JHL). D11.15 was chosen for this purpose because of significant homology in the residues immediately flanking the hypervariable domains of 118.1 and D11.15. The antigen binding loop of 118.1 HC CDR2 was predicted to consist of GYID-PYYGGITYN, with PYYG making the  $\beta$ -turn. Two libraries, each with five randomized residues in this region were expressed as N-terminal fusions with M13 gene III. To select for high affinity peptides, a monovalent phage display system was utilized in which one copy or less of the gene III protein is expressed as a fusion peptide on each phage (24). Phage expressing these libraries were panned on Mac-1 for four rounds with increasingly stringent wash conditions. Sequencing of phage eluted after four rounds revealed a consensus from one of the libraries, in which all five randomized residues differ from

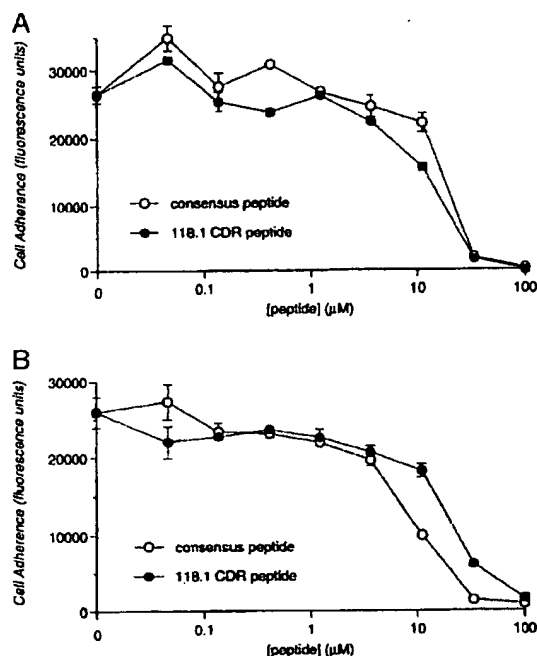


FIG. 4. Consensus peptide derived from phage display of 118.1 CDR blocks ICAM-1-CHO cell adherence. Mac-1 (A) or the I-domain (B) was preincubated with the 118.1 HC CDR2 peptide GYID-PYYGGITYNQIFKG or the phage derived variant GYRDGYAGPILYN-QIFKG for 15 min prior to the addition of ICAM-1-CHO cells. These data are the means of duplicate determinations ( $\pm$  individual values) and are representative of three (A) and two (B) experiments.

the original sequence: GYRDGYAGPILYN.

The phage-derived consensus sequence (plus five C-terminal residues from the 118.1 sequence) was synthesized and tested for inhibitory activity. The consensus peptide was equivalent to the corresponding 118.1 peptide in blocking the adherence of ICAM-1-CHO cells to Mac-1 (Fig. 4A) and to I-domain (Fig. 4B). Peptides made from the consensus sequence either with flanking residues from gene III or lacking the five added flanking residues from the 118.1 sequence were inactive (data not shown).

#### DISCUSSION

Peptides that block the binding of ICAM-1 to Mac-1 have been derived from the CDRs of anti-Mac-1 antibodies. Antibodies were chosen for this purpose based on their ability to block Mac-1-mediated adherence of ICAM-1-expressing CHO cells. The specificity of these antibodies was further verified by showing that they also block ICAM-1-CHO cell adherence to the recombinant I-domain of Mac-1. The amino acid sequences of the three HC CDRs and three light chain CDRs of each of the antibodies were deduced from the nucleotide sequences encoding the antibodies, and peptides were made from 15 of the 18 total CDR sequences. The adherence of ICAM-1-CHO cells to Mac-1 was blocked by four of these peptides, two from MY906 and one each from 44aabc and 118.1. This may result from one or two of the CDRs contributing most of the binding affinity of the antibody, or it may reflect a subpopulation of peptides taking on an active conformation out of their native context.

Because these peptides compete with ICAM-1 for binding to Mac-1, they were analyzed for homology with ICAM-1 (Ref. 25; Lasergene, DNASTar, Madison, WI). Some similarity was found between HC CDR2 from 118.1 and residues Val<sup>238</sup> to Ala<sup>249</sup> of ICAM-1 (Table II). These residues in ICAM-1 constitute the

TABLE II  
Alignment of 118 HC CDR2 with ICAM-1

ICAM-1	226HLALGDQRLNPTVTYGNDSFSAKA <sup>249</sup>
118.1 HC CDR2	EWIGYIDPYYGGITY-NQIFKGKA

loop between  $\beta$ -strands D and E in immunoglobulin domain 3. Mutations in loops between strands C and D and strands E and F of domain 3 have been shown to inhibit adhesion to Mac-1 (8).

A variant of HC CDR2 from 118.1 was identified using a monovalent display system on the phage M13 and selecting for Mac-1 binding. Interestingly, although all five randomized residues differed from the original 118.1 sequence, this peptide was found to possess similar Mac-1 blocking activity to the parental 118.1 peptide, indicating that there is considerable room for variation, and presumably improvement, within this CDR sequence. The selection of this CDR variant through binding of phage to Mac-1 demonstrates that this peptide does bind to Mac-1 and provides evidence that its blocking activity can be attributed to a specific interaction with Mac-1.

Evidence has suggested that protein-protein interaction involves multiple contact sites, and until recently little progress had been made in disrupting such interactions with small peptides. However, several examples now exist of peptides that can block protein-protein interaction; for example, an 8-amino acid peptide has been identified that can block the activation of FGFR1 by basic fibroblast growth factor (26). Further, the interaction of the two cell surface proteins, CD4 and MHC class II, can be blocked by a heptapeptide based on a protruding loop from one of the immunoglobulin-like domains of CD4. Thus even in this latter example, where many contact sites from two Ig domains of CD4 are thought to interact with MHC class II, binding appears to depend on a single  $\beta$ -turn-containing loop (27). In the case of antibody-antigen interaction, varying numbers of hypervariable regions may act together to provide the net affinity. In each of the three antibodies we have examined, we have identified one or two CDRs as candidates for important binding sites.

Peptides that block the interaction of ICAM-1 with Mac-1 have not previously been reported. Peptides derived from factor X and related peptides derived from filamentous hemagglutinin have been shown to prevent factor X binding to cells, presumably via Mac-1 (28, 29). However, the I-domain is not the primary binding site for factor X (12), and these peptides have not been shown to affect ICAM-1 binding. There is precedent, however, for peptide inhibition of ICAM-1- $\beta_2$ -integrin interaction. A peptide from ICAM-2 has been shown to bind to LFA-1 and inhibit endothelial cell adhesion (at 100  $\mu$ g/ml), presumably via ICAM-1 (30). Peptides from ICAM-1, domains 1 and 2, have also been shown to inhibit ICAM-1-mediated adhesion to unknown ligands (at 100  $\mu$ M) (31).

In conclusion, we have utilized sequence information from anti-Mac-1 antibodies to derive the first peptide Mac-1 antagonists. These findings support a growing body of evidence that protein-protein interactions may depend disproportionately on one site or pocket.

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## Systematic Exploration of the Antigen Binding Activity of Synthetic Peptides Isolated from the Variable Regions of Immunoglobulins\*

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Sets of short (12 residues) cellulose-bound synthetic overlapping peptides derived from the sequences of the variable regions of the heavy and light chains of three different antibodies (an anti-thyroglobulin antibody, the HyHEL-5 anti-lysozyme antibody, and an anti-angiotensin II antibody) were used to systematically assess the antigen binding capacity of peptides from the antibody paratope outside their natural molecular context. Peptides enclosing one or several of the complementarity determining region (CDR) residues had antigen binding activity, although the most active peptides were not necessarily those bearing the greatest number of CDR residues. Several residues from the framework region, preceding or following the CDR, were found to play a role in binding. Affinity constants from  $4.1 \times 10^{-7}$  to  $6.7 \times 10^{-8} \text{ M}^{-1}$  for the soluble form of 9 lysozyme-binding dodecapeptides were measured by BIAcore analysis. Alanine scanning of lysozyme-binding hexapeptides from the HyHEL-5 sequence identified 38 residues important for binding, of which 22 corresponded to residues that had been shown by x-ray crystallography to be at the interface between HyHEL-5 and lysozyme. Our results could be of interest for the rational identification of biologically active peptides derived from antibody sequences and in providing an experimental basis for mutagenesis of the antibody paratope.

Antibody molecules bind antigens with high affinity and specificity by synergistically using multiple noncovalent forces. The combining site (paratope), whose shape is complementary to the epitope on the antigen, is made up of the hypervariable regions, also called complementarity determining regions (CDRs)<sup>1</sup> (1). It is commonly accepted that there are three CDRs in the light chain (L1, L2, and L3) and in the heavy chain (H1, H2, and H3). These CDRs fold into turn structures that are stabilized by the  $\beta$ -sheet framework of the variable domains. The interface between antibodies and antigens has been precisely described by x-ray crystallographic studies, and several complexes between Fab fragments of monoclonal antibodies and peptide or protein antigens have been recently described

(for reviews see Refs. 2–4). The structures of antibody-antigen complexes indicate that at least four of the CDRs, and in some cases all six CDRs, contribute to antigen binding (5). Residues in the framework have rarely been reported to participate in this interaction (6, 7).

Antibody-peptide or antibody-protein complexes are excellent model systems to study the physicochemical requirements for molecular recognition. Unfortunately, it is a difficult task to obtain crystals suitable for the structural elucidation of antibody fragments in complex with proteins or peptides. Therefore, other approaches to obtain information about the key residues involved in the interaction would be very useful, in particular for paratope mutagenesis. Some workers have demonstrated that synthetic peptides derived from the amino acid sequences of CDRs bind antigens with specificities similar to those of the original antibody molecules (8–15). Such peptides have very often been chosen in the CDR3 of the  $V_H$  sequence, which is considered to play a prominent role in defining antibody reactivity. However, the capacity of synthetic peptides derived from the variable regions of a given antibody to bind an antigen has never been probed in a systematic manner, i.e. in assessing the antigen binding capacity of every overlapping peptide from the  $V_H$  and  $V_L$  sequences.

In this study, we present the results obtained by measuring the ability of an antigen to bind to sets of immobilized overlapping peptides of uniform size covering the amino acid sequences of the  $V_H$  and  $V_L$  domains of three different antibodies. The peptides were prepared by the Spot method (16), which has previously been successfully used to identify peptide epitopes recognized by anti-protein antibodies and further developed to map protein-protein interaction sites (17). Our results indicate that numerous peptides show antigen binding capacity, most of them exhibiting measurable affinities in BIAcore, and that paratope residues important for antigen recognition can be identified by peptide analysis.

### EXPERIMENTAL PROCEDURES

**Antigens**—Hen egg white lysozyme was from Sigma, and human thyroglobulin was from UCB-Pharma (Nanterre, France). Synthetic angiotensin II and an N-terminally biotinylated derivative were prepared as described below.

**Protein Biotinylation**—2 mg of the antigen in 2 ml of bicarbonate buffer (pH 8.6) were biotinylated by using a commercial reagent (Amersham RPN2202) according to the manufacturer's protocol. Biotinylated antigens were incubated with 0.1 M glycine (1 h, 37 °C) and then stored in phosphate-buffered saline at –20 °C.

**Amino Acid Sequences of Antibodies**—The numbering of the amino acid sequences of the variable regions was that of Wu and Kabat (1). The amino acid sequence of the anti-angiotensin II antibody 4D8 was established by sequencing the cDNAs corresponding to the heavy and light chains after reverse transcription of the mRNA from the 4D8 hybridoma (18). The amino acid sequences of the anti-lysozyme anti-

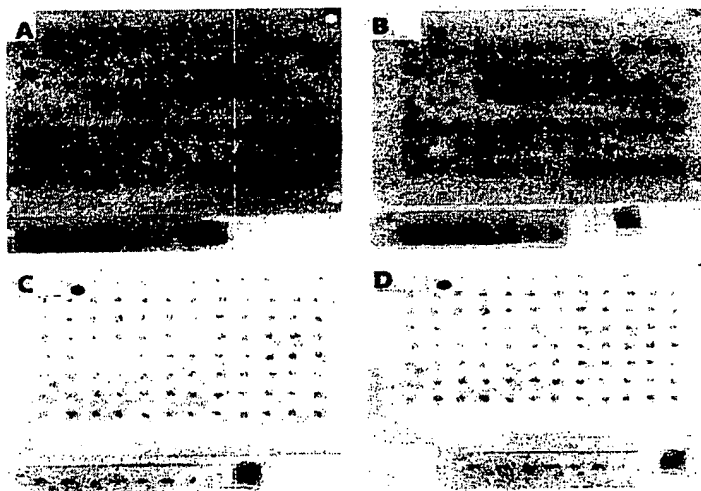
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The abbreviations used are: CDR, complementarity determining region;  $V_H$ , variable region of the heavy chain;  $V_L$ , variable region of the light chain; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography.



**FIG. 1. Reactivity of overlapping dodecapeptides derived from the sequence of the anti-lysozyme antibody HyHEL-5 with biotinylated lysozyme.** The membrane on which the peptides were synthesized was incubated with 1  $\mu$ g/ml biotinylated lysozyme (A), 1  $\mu$ g/ml biotinylated lysozyme in the presence of human normal serum diluted 1:50 (B), 1  $\mu$ g/ml biotinylated lysozyme in the presence of 0.1 mg/ml nonbiotinylated lysozyme (C), or alkaline phosphatase-streptavidin (1:3000) (D).



body HyHEL-5 and the anti-human thyroglobulin Tg10 antibody were taken from the literature (19, 20).

**Peptide Synthesis on Cellulose Membrane**—The general protocol has been described previously (21). Membranes were obtained from Abimed (Langenfeld, Germany). Fmoc amino acids and *N*-hydroxybenzotriazole were from Novabiochem. An ASP222 robot (Abimed) was used for the coupling steps. All peptides were acetylated at their N terminus. After the peptide sequences had been assembled, the side-chain protecting groups were removed by trifluoroacetic acid treatment (16).

**Immunoassay with Cellulose-bound Peptides**—The general protocol was the same as for epitope analysis (16) except for the use of a biotinylated antigen (90-min incubation at 37 °C). After washing the membrane, a 1:3000 dilution of an alkaline phosphatase-streptavidin conjugate (Sigma) was incubated for 30 min at room temperature. Binding was revealed by addition of a phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate-3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma), giving a blue precipitate on those spots having bound the alkaline phosphatase-streptavidin conjugate.

A plot of spot intensities was obtained with the NIH Image software after scanning the membrane as described (21). Color intensities were calculated with reference to a black spot taken as the maximum of a 0–255 scale (arbitrary units). To allow the reuse of the membrane, it was sequentially treated by dimethylformamide, 6 M urea, and 10% acetic acid in ethanol so as to remove the precipitated dye and molecules bound to peptides. The reactivity of each antigen was assessed in two or three independent experiments.

**Synthesis of Soluble Peptides**—All soluble peptides were synthesized on an Abimed AMS 422 synthesizer by Fmoc chemistry. Except for angiotensin II, a spacer sequence (YKK) was added at the N terminus of every peptide followed by a biotin residue. Peptides were deprotected and released from the resin by trifluoroacetic acid treatment in the presence of appropriate scavengers. Peptides were lyophilized, and their purity was assessed by HPLC. When necessary, peptides were purified to greater than 90% HPLC homogeneity.

**Real Time Analysis of Peptide-Antigen Interaction by BIAcore**—The BIAcore apparatus was from BIAcore (Uppsala, Sweden). All experiments were carried out at 25 °C. N-terminally biotinylated peptides (5  $\mu$ g/ml in Hepes-buffered saline buffer) were immobilized on a streptavidin-coated sensor chip. The injection was performed at a flow rate of 5  $\mu$ l/min. The net surface plasmon resonance signal for immobilized peptides was found to be about 25–30 resonance units after completion of the chip regeneration cycle, which corresponds to 25–30 pg/mm<sup>2</sup> (12–15 fmol/mm<sup>2</sup>). The binding kinetics of lysozyme to immobilized peptides was determined by injecting lysozyme (2–4  $\mu$ M) in Hepes-buffered saline buffer (running buffer) at a flow rate of 10  $\mu$ l/min. Dissociation was observed in running buffer without dissociating agents at a flow rate of 10  $\mu$ l/min. The kinetic parameters of the binding reactions were determined using BIAevaluation 2.1 software (22). The dissociation rate (off-rate) constant  $k_d$  was determined from a plot of  $\ln(R_d/R)$  versus time,  $R$  being the surface plasmon resonance signal at time  $t$ ; the association rate constant (on-rate)  $k_a$  was determined from a plot of  $\ln[ab/(dR/dt)]$  versus time. The apparent equilibrium dissociation constant was calculated from the kinetic constants:  $K_D = k_d/k_a$ .

**Analysis of Framework-CDR Interaction by Molecular Modeling**—

The coordinates of the HyHEL-5-lysozyme complex (3hfl; Ref. 23) were used with the Insight II software to identify CDR residues in contact (i.e. less than 3.4 Å apart) with amino acids from the framework that were found to be important by Spot peptide analysis.

## RESULTS

**Capacity of Peptides Derived from the  $V_H$  and  $V_L$  Sequences of Three Different Antibodies to Specifically Bind the Cognate Antigen**—The capacity of short peptides derived from the variable regions of three different antibodies to bind the cognate antigen was investigated in a systematic manner. The  $V_H$  and  $V_L$  amino acid sequences were presented as sets of 110 overlapping dodecapeptides (2-residue frameshift) synthesized according to the Spot method (16, 21). In this method, the peptides remain attached to the cellulose membrane used for their synthesis, and their immunoreactivity is probed by incubating the membrane with a solution containing the ligand. As an example, Fig. 1 shows the results obtained with peptides derived from the variable regions of HyHEL-5, an anti-lysozyme antibody (6). Biotinylated lysozyme (1  $\mu$ g/ml;  $6 \times 10^{-8}$  M) bound to several peptides derived from the  $V_H$  and  $V_L$  sequences of HyHEL-5 (Fig. 1A). A detailed analysis of this interaction is provided under "Relationships between the Sequence of Antigen-binding Peptides and CDR Location in the HyHEL-5 Model." The binding pattern was not affected by incubating the biotinylated lysozyme in a 50-fold dilution of normal human serum, i.e. in the presence of a high concentration of proteins unrelated to lysozyme (Fig. 1B). However, when lysozyme (100  $\mu$ g/ml) was added to the incubation mixture, binding no longer occurred (Fig. 1C). No binding was observed with the alkaline phosphatase-streptavidin complex (Fig. 1D) except on two control peptides that have the HPQ sequence recognized by streptavidin (24). The binding of lysozyme to immobilized peptides is therefore specific.

In the second model studied, biotinylated thyroglobulin (1  $\mu$ g/ml;  $3 \times 10^{-9}$  M) was also found to bind several peptides from the  $V_H$  and  $V_L$  domains of the anti-thyroglobulin antibody Tg10 (25). The reactivity was observed in six regions of the membrane that broadly corresponded to peptides containing CDR residues (data not shown). The reactivity was abolished in the presence of an excess of thyroglobulin.

In another set of experiments, the  $V_H$  and  $V_L$  domains of the high affinity anti-angiotensin II antibody 4D8 ( $K_D = 1.3 \times 10^{11}$  M; 18) were scanned by overlapping dodecapeptides. Biotinylated angiotensin II (1  $\mu$ g/ml;  $1 \times 10^{-6}$  M) bound to peptides corresponding broadly to the three CDRs of  $V_L$  and to H1, whereas peptides corresponding to H2 and H3 showed less

TABLE I

Sequences of the dodecapeptides derived from the V<sub>H</sub> and V<sub>L</sub> of antibody HyHel-5 and their reactivity with biotinylated lysozyme. Framework residues are colored in black, and CDR residues are in red. Shaded areas correspond to peptides showing significant binding.

Peptide number	Peptide sequence	Color intensity of the spots (±SD)
2	AMKLPQPG CONTROL PEPTIDE	232 (±3)
3	DTVLTSPAINK CDR L1	32 (±5)
4	VLTPSPAINK	35 (±2)
5	TPSPAINK	26 (±4)
6	SPAINKSPK	40 (±2)
7	ATKASPKGVY	40 (±2)
8	TKASPKGVY	39 (±1)
9	AKSPGVY	35 (±4)
10	KSPGVY	43 (±4)
11	SPGVY	36 (±4)
12	VTMTCSASSSVN	41 (±4)
13	MTCSASSSVNYM	193 (±5)
14	CSASSSVNYM	243 (±8)
15	ASSSVNYM	130 (±20)
16	SSVNYM	120 (±22)
17	VYNYM	105 (±7)
18	YNYM	145 (±4)
19	NYM	151 (±7)
20	YQKSTSPKRN CDR L2	47 (±14)
21	QKSTSPKRN	61 (±9)
22	STSPKRN	225 (±5)
23	SPKRN	171 (±4)
24	PKRN	142 (±14)
25	KRN	134 (±5)
26	ITDKLASQVF	75 (±4)
27	DKLASQVF	71 (±8)
28	KLASQVF	57 (±2)
29	LASQVF	154 (±3)
30	ASQVF	207 (±5)
31	VYVYKSGQST	194 (±11)
32	VYVYKSGST	225 (±8)
33	YVYKSGST	235 (±10)
34	YKSGST	47 (±3)
35	KGST	52 (±2)
36	GTSTLTSSK CDR L3	60 (±5)
37	STLTSSK	56 (±4)
38	TLTSSK	76 (±2)
39	LTSSK	36 (±6)
40	SKSTDAATY	227 (±3)
41	KSTDAATY	200 (±4)
42	STDAATY	243 (±2)
43	DAATY	252 (±3)
44	AATY	242 (±8)
45	YTCQGRNTP	244 (±10)
46	TCQGRNTP	250 (±3)
47	CQGRNTP	244 (±2)
48	QGRNTP	99 (±8)
49	GRNTP	104 (±7)
50	RNTP	50 (±10)
51	VLQGSQAKLK CDR H1	35 (±5)
52	LQGSQAKLK	34 (±3)
53	QGSQAKLK	44 (±3)
54	GSQAKLK	48 (±4)
55	SQAKLK	43 (±4)
56	QAKLK	44 (±3)
57	AKLK	49 (±1)
58	KLK	48 (±2)
59	YLKCKASCTY	121 (±8)
60	LKCKASCTY	235 (±4)
61	KCKASCTY	253 (±3)
62	CKASCTY	241 (±2)
63	ASCTY	76 (±5)
64	TSSTYKRVK CDR H2	235 (±7)
65	SSTYKRVK	225 (±10)
66	STYKRVK	62 (±8)
67	TYKRVK	129 (±5)
68	YKRVK	212 (±9)
69	KRVK	62 (±5)
70	RVK	129 (±5)
71	VK	106 (±7)
72	K	213 (±4)
73	IK	160 (±3)
74	IKLQKGRN	149 (±10)
75	KLQKGRN	237 (±3)
76	LQKGRN	187 (±2)
77	QKGRN	60 (±5)
78	KGRN	69 (±2)
79	GRN	82 (±6)
80	RN	62 (±3)
81	N	54 (±2)
82	YTPADTSSA CDR H3	49 (±3)
83	TPADTSSA	44 (±2)
84	PADTSSA	150 (±16)

TABLE I—continued

85	ADTESPAINK CDR H3	74 (±3)
86	TESPAINK	77 (±3)
87	SPAINK	145 (±10)
88	PAINK	104 (±2)
89	AINK	175 (±5)
90	INK	48 (±3)
91	K	235 (±5)
92	LTSTSDGVY	202 (±5)
93	TSTSDGVY	236 (±7)
94	STSDGVY	238 (±3)
95	TSDGVY	207 (±4)
96	SDGVY	254 (±1)
97	DGVY	252 (±2)
98	GVY	244 (±1)
99	Y	249 (±1)
100	YQKSTSPKRN	112 (±9)
101	QKSTSPKRN	96 (±5)
102	STSPKRN	225 (±5)

with any of the three antigens on peptides derived from unrelated antibodies. All of these results showed that 12-mer peptides derived from the amino acid sequence of the V<sub>H</sub> and V<sub>L</sub> domains of three different antibodies are capable of being specifically recognized by their cognate antigen.

**Relationships between the Sequence of Antigen-binding Peptides and CDR Location in the HyHEL-5 Model**—Table I shows the results of the quantitative analysis of the binding of biotinylated lysozyme to cellulose-bound peptides derived from the amino acid sequence of HyHEL-5. The majority of peptides containing only framework residues displayed no binding activity (peptides 3–8, 20–21, 34–39, 51–58, 84, and 86–87). However, several peptides (31–33, 40, 59, 68–69, 85, 88–90, and 92–93) that also contained only framework residues were reactive. The possible molecular basis for this reactivity is discussed under "Precise Identification of Residues Contributing to Antigen Binding and Comparison with Contact Residues Defined by X-ray Crystallography". Analysis of the relationships between the amino acid sequence and the binding properties (Table I) indicated that strong binding capacity was detectable when certain CDR residues were present in the peptide sequence. For example, the sequence VTMTCSASSSVN from L1 (peptide 12) had no activity, but the following (overlapping) sequence MTCSASSSVNYM (peptide 13) possessed activity; therefore, the motif YM clearly contributed to binding. Other residues from CDRs were thus identified as contributors: DT from L2 at peptide 22, SD from H1 at peptide 60, and YH from H2 at peptide 75. Some residues not belonging to CDRs also apparently contributed to binding activity: GS at peptide 29, YY at peptide 40, TF at peptide 59, KQ at peptide 64, GL at peptide 67, LT at peptide 88, and YY at peptide 92. Also, a decrease in the binding capacity of certain peptides occurred when residues from the CDRs disappeared from the peptide sequence, e.g. the difference in the activities of peptides 19 and 20 could be attributed to the absence of YW in peptide 20. The following CDR residues were thus identified: QW from L3 (difference in binding to peptide 47 versus 48), SD from H1 (peptide 65 versus 66), GS from H2 (peptide 77 versus 78), and DF from H3 (peptide 101 versus 102). When certain residues not belonging to the CDR were removed from the amino acid sequence of a binding peptide, the antigen binding capacity was reduced: absence of RW in the sequence of peptide 26, of FS in peptide 34, of AS in peptide 63, of EW in peptide 74, and of YM in peptide 91. Therefore, it seems that the binding of antigen to cellulose-bound peptides is based on the presence in their sequence of certain residues from the CDRs and in several instances of certain framework residues neighboring the CDRs.

**Precise Identification of Residues Contributing to Antigen Binding and Comparison with Contact Residues Defined by X-ray Crystallography**—Alanine scanning of hexapeptides derived from each previously identified binding sequence from

intense reactivity (not shown). When biotinylated angiotensin II (1 µg/ml) was incubated with 1 mg/ml angiotensin II, the signal markedly decreased. No cross-reactivity was observed

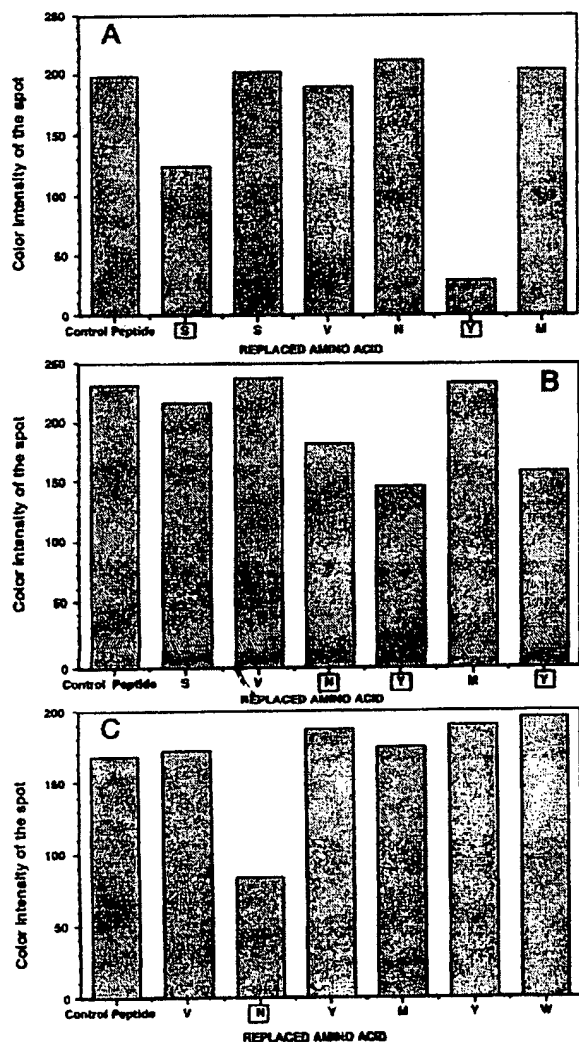


FIG. 2. Determination of residues contributing to the binding of biotinylated lysozyme to sets of alanine analogs of hexapeptides derived from the L1 sequence of HyHEL-5 antibody. Each bar represents the reactivity of the hexapeptide whose sequence comprises an Ala residue in place of the indicated amino acid. Boxed letters indicate amino acids considered as contributors. A, alanine scanning of peptide SSVNYM. B, alanine scanning of peptide SVNYMY. C, alanine scanning of peptide VNYMYW.

the  $V_H$  and  $V_L$  domains of HyHEL-5 was performed to identify the exact residues contributing to antigen binding. The study of the CDR L1 region is given as a detailed example (Fig. 2; see legend of Fig. 3 for the amino acid numbering of the  $V_H$  and  $V_L$  sequences). Three hexapeptides (SSVNYM, SVNYMY, and VNYMYW) and each of their six alanine analogs were synthesized by the Spot method and tested for reactivity with biotinylated lysozyme. Replacing Ser<sup>27</sup> of peptide SSVNYM by an alanine residue led to a fall in the antigen binding capacity, whereas alanine replacement of Tyr<sup>32</sup> led to disparition of the binding. Changing any of the other amino acids of the peptide SSVNYM did not modify the binding (Fig. 2A). For peptide SVNYMY, the three amino acids Asn<sup>31</sup>, Tyr<sup>32</sup>, and Tyr<sup>34</sup> appeared to be important (Fig. 2B). Alanine scanning of peptide VNYMYW indicated that only Asn<sup>31</sup> is important (Fig. 2C). In preliminary experiments, we noted that the contribution of a given amino acid to binding was not always the same when the

surrounding sequence varied, probably reflecting conformational effects. Indeed, Tyr<sup>32</sup> appeared to be a contributor in peptides SSVNYM and SVNYMY but not a contributor in peptide VNYMYW; also, the influence of Asn<sup>31</sup> was apparently more critical in sequence VNYMYW than in other sequences. In fact, several hexapeptides had to be analyzed to define the residues contributing to the binding.

The contributing motif for the L1 region was therefore determined to be <sup>27</sup>S-NY-Y<sup>34</sup> (Fig. 3), with all four residues belonging to the CDR. Using the same alanine-scanning approach, contributors were determined for each CDR region. For the L2 region, the motif was identified as <sup>48</sup>RWYID<sup>50</sup> (Fig. 3); one residue (Asp<sup>50</sup>) belonged to the CDR, and four residues were from the framework sequence preceding L2. For the L3 region, the contributing motif was <sup>91</sup>WGR-P-F<sup>98</sup> (Fig. 3). Residues Trp, Gly, Arg, and Pro were part of the CDR, Phe<sup>98</sup> being in the framework. For the H1 region, the contributing motif <sup>27</sup>Y-FSDYW-EW<sup>36</sup> comprised four residues (DYW-E) from the CDR and four residues from the framework. For the H2 region, the motif comprised a framework residue, Trp<sup>47</sup>, and six residues in the middle of the CDR (<sup>64</sup>S-S-NYHE<sup>61</sup>). For the H3 region, the motif <sup>91</sup>YC-HGNYDF-W<sup>103</sup> had five amino acids from the CDR (GNYDF) and four residues from the framework (YC-H and Trp, respectively on the N- and C-terminal sides of the CDR). The determination of critical residues for binding the antigen provided a possible explanation for the reactivity of certain framework sequences; for example, peptides 29–34 (Table I) contain an SGS sequence that is part of the CDR H2; peptides 68 and 69, could be reactive because of the presence of the LEW sequence very similar to the IEW motif of H1. The reactivity of peptide 88 could be due to a strong sequence similarity with peptides 16–18 from the L1 region (SSTAYM as compared with SSVNYM). Thus, a cross-reactivity phenomenon may explain some of the reactivities in framework peptides; however, no similar explanation for the reactivity of peptides 40, 59, 89–90, and 92–93 was found.

The residues identified here as important for the binding of lysozyme to peptides derived from the sequence of the  $V_H$  and  $V_L$  domains of HyHEL-5 were then compared with the residues involved in antigen binding in the crystal structure of HyHEL-5 Fab-lysozyme (6, 23) (Fig. 3). For the L1 region, three amino acids (NY-Y) identified by peptide analysis are implicated in the crystallographic antigen-antibody interaction; Ser<sup>27</sup> was found to be a contributor in our analysis only. For the L2 region, only one CDR amino acid (Asp<sup>50</sup>) was implicated by crystallography in the antigen-antibody complex. Alanine scanning of reactive peptides from this region pointed out the role of both Asp<sup>50</sup> and of the stretch of four residues preceding it. For the L3 region, the <sup>91</sup>WGR-P-F<sup>98</sup> residues of the antigen-antibody complex were identified by peptide analysis with, however, the implication of an additional residue (Phe<sup>98</sup>). For the H1 region, four CDR residues (DYW-E) and one framework residue (Ser<sup>30</sup>) were found by crystallography to be implicated in antigen recognition; by alanine scanning of hexapeptides, the same five amino acids, <sup>30</sup>SDYW-E<sup>38</sup>, were found to be contributors. However, three other amino acids (Y-F-W) outside the CDR seemed to be implicated in the interaction of the peptide with the antigen. For the H2 region, which is a 17-residue long CDR, seven amino acids from the CDR and one in the framework play a role in the antigen-antibody interaction as determined by x-ray crystallography. By using the Spot method, the importance of three amino acids of the CDR (Ser<sup>54</sup>, Ser<sup>56</sup>, and Asn<sup>58</sup>) and of the Trp<sup>47</sup> residue of the framework was determined. Whereas the crystallographic results implicate the N-terminal part and the middle of the CDR, the C-terminal part of the CDR (motif YHE) seemed to play an important role when peptides

	L1 region	L2 region	L3 region
Contributing residues found by alanine scanning with hexapeptides	<sup>27</sup> S-- <sup>34</sup> NY-Y	<sup>46</sup> RW <sup>50</sup> IYD---	<sup>91</sup> -WGR-P- <sup>98</sup> F
Residues in the Ag-Ab interface (crystallography)	<sup>1</sup> DI- <sup>2</sup> SV <sup>3</sup> NY-Y	---- <sup>53</sup> D--K	<sup>90</sup> QWGR-P--

	H1 region	H2 region	H3 region
Contributing residues found by alanine scanning with hexapeptides	<sup>27</sup> Y-FSDYW- <sup>36</sup> EW	<sup>47</sup> W----- <sup>61</sup> S-S-NYHE	<sup>91</sup> YC--HGNYDF-- <sup>103</sup> W
Residues in the Ag-Ab interface (crystallography)	---SDYW-E-	W--E-L--SGSTNY--	-----GNYD----

FIG. 3. Comparison of residues found to contribute to the binding of lysozyme to peptides with residues involved in the paratope-epitope interface in the HyHEL-5 lysozyme complex (23). CDR residues are colored in red. Residues labeled with asterisks correspond to residues in direct contact with lysozyme in the crystal structure. A residue was considered to be a contributor if the binding signal was reduced by at least 20% when it was replaced by an alanine. Ab, antibody. The complete amino acid sequences of the V<sub>H</sub> and V<sub>L</sub> of HyHEL-5 are given below. CDR residues are underlined.

VL  
1 5 10 15 20 25 29 35 40 45 50 55 60 70 75 80 85 90 94 100 105  
DIVLTQSPAIMSASPGEKVTMTCSASSSVNYMYWYQQKSGTSPKRWIYDTSKLASGVVPRFSGSGSGTSYSLTISMETEDAAEYICQOWGRNPTFGGGTKLEIK

VH  
1 5 10 15 20 25 30 35 40 45 50 52a 55 60 65 70 75 80 82abc 85 90 95  
ZVQLQSGAELMKPGASVKISCKASGYTFS<sup>91</sup>DY<sup>95</sup>WIEWVKRPGHGLEWIGEI L PGSGSTNYHERFKGKATFTADTSSSTAYM Q LNSLTSEDGSGVYCLH-  
99 105 110  
GNYDFDGGWGQGTTLTVSS

## SEQUENCES 1 AND 2

were used. For the H3 region, the motif GNYD found by crystallography is similar to the motif found by the alanine scanning of hexapeptides. However, five amino acids, which were defined by spot peptide analysis as contributors, were not implicated in the HyHEL-5-lysozyme interaction as defined in the crystal structure. Of these five contributors, four of them belonged to the framework (<sup>91</sup>YC-H<sup>95</sup>, N-terminal to the CDR, and Trp<sup>103</sup>, C-terminal to the CDR).

The comparison between residues (totaling 38) important for lysozyme binding to V<sub>H</sub>- and V<sub>L</sub>-derived peptides and residues involved in HyHEL-5-lysozyme interaction permitted us to identify 14 of the 18 residues in direct crystallographic contact with the antigen (78%) and 22 of the 32 (69%) residues that are either in contact or partially buried at the antigen-antibody interface (23) (Fig. 3). Sixteen residues that were found to be important in the binding of biotinylated lysozyme to peptides did not correspond to amino acids at the antigen-antibody interface. The majority of them (12 of 16) were framework residues. Table II shows that all five contributors from the V<sub>L</sub> framework and six of nine important residues from the V<sub>H</sub> framework belong to the subset of framework residues from the "Vernier" zone, i.e. defined as residues that may adjust CDR structure and fine tune the fitting to antigen (26). By using the atomic coordinates of the HyHEL-5-lysozyme complex, it was found that, except for Tyr<sup>91H</sup> and Cys<sup>92H</sup> (not in the Vernier zone), all the other framework residues identified as contributors to antigen binding by peptide analysis make contacts in the crystal structure with at least adjacent residues from the CDR (Table II). Therefore, this subset of residues could contribute by giving the peptide an appropriate conformation for

TABLE II  
Characterization of framework residues found to be important for antigen binding by peptide analysis

Contributing residue from the framework	Belonging to the Vernier zone <sup>a</sup>	Contact residue from the adjacent CDR (CDR) <sup>b</sup>
Arg <sup>46L</sup>	Yes	Ala <sup>96L</sup> (CDR L2)
Trp <sup>47L</sup>	Yes	Leu <sup>54L</sup> , Ala <sup>55L</sup> (CDR L2)
Ile <sup>48L</sup>	Yes	Thr <sup>51L</sup> , Ser <sup>52L</sup> , Lys <sup>53L</sup> (CDR L2)
Tyr <sup>49L</sup>	Yes	Asp <sup>50L</sup> , Lys <sup>51L</sup> , Leu <sup>54L</sup> , Ala <sup>55L</sup> (CDR L2)
Phe <sup>90L</sup>	Yes	Thr <sup>97L</sup> , Gln <sup>89L</sup> , Pro <sup>96L</sup> (CDR L3)
Tyr <sup>27H</sup>	Yes	Tyr <sup>32H</sup> (CDR H1)
Phe <sup>29H</sup>	Yes	Asp <sup>31H</sup> , Tyr <sup>32H</sup> (CDR H1)
Ser <sup>30H</sup>	Yes	Asp <sup>31H</sup> (CDR H1)
Trp <sup>36H</sup>	Yes	Ile <sup>34H</sup> , Glu <sup>35H</sup> (CDR H1)
Trp <sup>47H</sup>	Yes	Glu <sup>50H</sup> , His <sup>60H</sup> (CDR H2)
Tyr <sup>91H</sup>		
Cys <sup>92H</sup>		
His <sup>94H</sup>	Yes	G95H, N96H, F100H, D101H (CDR H3)
Trp <sup>103H</sup>	Yes	D101H, G102H (CDR H3)

<sup>a</sup> Ref. 26.

<sup>b</sup> Contact as defined under "Experimental Procedures." The list of contacts is not exhaustive.

antigen recognition.

**Affinity Determination of the Interaction between Peptides and Lysozyme by BIAcore Analysis**—Based on the sequences of the peptides used in the Spot assay, a series of peptides derived from the variable regions of HyHEL-5 was synthesized by conventional solid phase synthesis and used in BIAcore real time interaction analysis (27). Fig. 4A shows a typical sensorgram of the interaction of an immobilized biotinylated peptide (Lyso 1) with lysozyme; the fitting of the experimental associ-

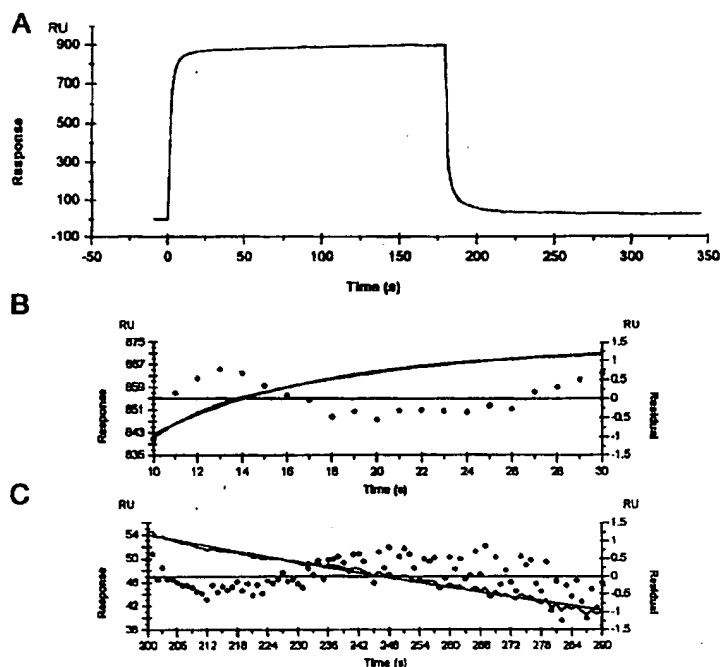


FIG. 4. Surface plasmon resonance analysis of interaction between lysozyme and peptide Lyso 1. A, sensorgram for the binding of lysozyme to peptide Lyso 1. B, on-rate constant determination using a one-site model with data from A; the experimental and theoretical curves and the residuals between these two curves are overlaid. C, off-rate constant determination using a one-site model with data from A; the experimental and theoretical curves and the residuals between these two curves are overlaid. RU, resonance units.

ation curve (Fig. 4B) and dissociation curve (Fig. 4C) with the theoretical curves indicated that a one-site interaction model satisfactorily described the binding. The same observations were made for all other measurable peptide interactions (not shown). Table III summarizes the results of the BIAcore study in which the kinetic parameters  $k_a$  and  $k_d$  were measured. Among 10 soluble peptides corresponding to sequences highly reactive in the Spot assay, at least one peptide from each of the six hypervariable regions of the HyHel-5 antibody was found to exhibit measurable binding with a  $K_D$  value in the  $0.6\text{--}4 \times 10^{-7}$  M range (upper part of Table III). Two other soluble synthetic peptides (Lyso 15 and Lyso 17) showed no measurable binding in BIAcore analysis. For Lyso 6, Lyso 10, and Lyso 16, corresponding to peptides from the framework of HyHEL-5 exhibiting binding activity in Spot, only Lyso 6 displayed an affinity constant that was similar to that measured for the other peptides. As expected, irrelevant peptides or peptides derived from sequences of faintly or noncolored spots gave no binding in the BIAcore assay (bottom part of Table III). These results indicate that several short (12 residues), linear peptides identified on the basis of their reactivity in the Spot format gave rise to interaction of sufficient energy with lysozyme to be analyzed by real time interaction analysis. However, there were some peptides reactive in the Spot assay that did not show measurable affinity, making it difficult to infer BIAcore behavior from the results of the Spot analysis.

#### DISCUSSION

Antigen binding occurs through molecular contacts with several of the spatially juxtaposed CDRs of the  $V_H$  and  $V_L$  domains of the antibody molecule (28, 29). A systematic evaluation of the capacity of every peptide from the  $V_H$  and  $V_L$  regions to bind the antigen was performed in this study to assess the effect of keeping the sequence information but disrupting the precise molecular arrangement of the paratope. It was found that in the case of three different antigens (angiotensin II, hen egg white lysozyme, and human thyroglobulin), differing considerably in their size, numerous immobilized peptides from the  $V_H$  and  $V_L$  regions of the cognate antibody bind the antigen

in a specific manner. Moreover, we have unpublished results showing that in the case of two other anti-protein antibodies the same observations could be made, arguing in favor of the generality of the phenomenon for anti-protein antibodies. Our present analysis indicates that peptides with antigen binding activity have one or several residues from the CDR in their amino acid sequence. Synthetic peptides enclosing complete CDRs generally displayed strong binding activity; however, peptides representing incomplete CDRs but including amino acids from sequences flanking the CDRs were also active, indicating contribution to the binding of some residues outside the CDR itself. It is not clear whether these residues contribute by directly contacting the antigen or by giving the reactive conformation to the peptide. We observed, however, that these residues often belong to the subset of framework residues that could modulate the conformation of the adjacent CDR (19, 26). It is therefore possible that the conformational state of reactive peptides immobilized on the cellulose membrane is very close to the conformation of the same sequence in the paratope. If this is the case, modification of a peptide residue critical for conformation would affect antigen binding in the same way that mutation of the corresponding framework residue would affect binding by altering CDR conformation. It was observed that some peptides including only framework residues do specifically bind to the antigen; this is possibly due in certain cases to occurrence in the framework sequence of similarities with the motifs contributing to antigen binding. It has been shown that a sequence homology of three residues in a peptide is sufficient to give rise to antigenic cross-reactivity (30). However, in several instances there is no obvious homology so that the chemical or structural basis for this reactivity remains to be assessed.

Binding of nine of thirteen synthetic peptides corresponding to those active in the Spot assay was observed by BIAcore real time interaction analysis. The dissociation constants of the interaction between soluble peptides and lysozyme in the 70–400 nm range are only 2 or 3 orders of magnitude higher than the  $K_D$  of the reaction between the whole antibody and ly-

TABLE III  
Determination of the binding kinetics of the interaction between lysozyme and biotinylated peptides derived from the V<sub>H</sub> and V<sub>L</sub> sequences of HyHEL-5

Name of the peptide	Peptide sequence	Color intensity <sup>a</sup>	Corresponding antibody region	$k_a$ $10^4 \text{ s}^{-1} \text{ M}^{-1}$	$k_d$ $10^{-3} \text{ s}^{-1}$	$K_D$ $10^{-7} \text{ M}$
Lyso 1	Biot-Sp-CSASSSVNYMYW	192	CDR L1	3.22	3.84	1.19
Lyso 3	Biot-Sp-SGTSPKRWIYDT	201	CDR L2	5.78	3.89	0.673
Lyso 4	Biot-Sp-RWIYDTSKLASG	171	CDR L2	2.38	6.27	0.857
Lyso 7	Biot-Sp-YYCQWGRNPTF	180	CDR L3	2.33	3.37	1.45
Lyso 8	Biot-Sp-CKASGYTFSQYW	197	CDR H1	1.10	4.54	4.12
Lyso 9	Biot-Sp-TFSDYWLWVKQ	191	CDR H1	1.33	3.70	2.78
Lyso 11	Biot-Sp-RPGHGLEWIGEI	203	CDR H2	1.99	3.19	1.60
Lyso 18	Biot-Sp-CLHGNYDFDQWG	254	CDR H3	2.69	3.67	1.36
Lyso 15	Biot-Sp-LPGSGSTNYHER	195	CDR H2	NM <sup>b</sup>	NM	NM
Lyso 17	Biot-Sp-GVYVCLHGNYDF	203	CDR H3	NM	NM	NM
Lyso 6	Biot-Sp-SSMETEDAAEYY	209	FRW	4.85	7.39	1.52
Lyso 10	Biot-Sp-WVKQRPQHGLEW	182	FRW	NM	NM	NM
Lyso 16	Biot-Sp-NSLTSEDSGVYY	220	FRW	NM	NM	NM
Lyso 2	Biot-Sp-SSVNYMYWYQQK	84	CDR L1	NM	NM	NM
Lyso 5	Biot-Sp-IYDTSKLASGVP	35	CDR L2	NM	NM	NM
Lyso 12	Biot-Sp-EWIGEILPGSGS	57	CDR H2	NM	NM	NM
Lyso 13	Biot-Sp-IGBILPGSGSTN	40	CDR H2	NM	NM	NM
Lyso 14	Biot-Sp-EILPGSGSTNYH	77	CDR H2	NM	NM	NM
Lyso 19	Biot-Sp-LQQSGAELMKPG	32	FRW	NM	NM	NM
Lyso 20	Biot-Sp-GKATFTADTSSS	35	FRW	NM	NM	NM
	Biot-Sp-AngiotensinII	ND	IRREL.	NM	NM	NM
	Biot-Sp-GAD-peptide	ND	IRREL.	NM	NM	NM

<sup>a</sup> The color intensities of the spots were measured after incubation of the membrane with biotinylated lysozyme at 0.1  $\mu\text{g}/\text{ml}$ . ND, not determined.

<sup>b</sup> NM, not measurable.

sozyme (31). The kinetics of binding were characterized by rapid dissociation rates, probably due to a small number of interactions between the peptide and the antigen. To improve the binding capacity of such peptides derived from antibody sequences, it is suggested that cyclization could be useful in locking an active conformer (14, 32). Four peptides that were active in the Spot assay did not give measurable affinities in the BIAcore analysis, indicating that detection of antigen binding could be facilitated by the high density of peptides at the surface of the paper (about 3–11 nmol/mm<sup>2</sup>; Ref. 16), which is not the case in the BIAcore format where the peptide density is as low as 10 fmol/mm<sup>2</sup>.

The identification of short amino acid sequences recognized by peptide/protein antigens may have important applications for the screening of bioactive peptides based upon antibody sequences (11) in the current trend to redefine the minimal antigen-binding fragment (33) with the intention of preparing miniantibodies (34). Our systematic approach provides a way to select, on an experimental basis, peptide sequences with antigen binding activity. The results indicate that such sequences do not always correspond to peptides containing only CDR residues; in fact, residues from the framework were here found to play a critical role in the activity of CDR-based peptides, extending previous observations (13). Several residues that were found to contribute to peptide-lysozyme interaction were not residues located at the paratope-epitope interface defined by x-ray crystallography. They were mainly aromatic residues, suggesting that this type of residue plays a particular role in the efficiency of the interaction mechanisms. The role of tyrosine or phenylalanine residues in increasing the binding properties of cyclic peptides based on a CDR-like sequence of the CD4 molecule has been noticed (14, 15).

The identification of residues contributing to the binding of the antigen to peptides was performed in the HyHEL-5 antibody model for which detailed three-dimensional information on the interface between the antibody paratope and the antigenic protein is available (6, 23). Our identification was made in a blind manner and further compared with the results of

x-ray crystallography. 22 of the residues determined as important in peptides for binding biotinylated lysozyme belonged to the set of 32 residues in contact with the antigen or buried in the paratope-epitope interface (6, 23), indicating that the binding that was observed with short peptides was consistent with the binding of the antigen to the whole antibody. The capacity of the method we used here to pinpoint residues potentially involved in the antigen-antibody interface could be valuable for the design of experiments aimed at mutating antibody-binding sites; in this context, not knowing exactly whether residues identified as important play a structural or functional role would not be detrimental. We suggest that peptide analysis conjugated with powerful binding site topography prediction methods (35, 36) could be a valuable strategy for antibody engineering.

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